Elsevier Editorial System(tm) for Phytomedicine

Manuscript Draft

Manuscript Number: PHYMED-D-15-00774R2

Title: Proanthocyanidins from Vitis vinifera inhibit oxidative stress-induced vascular impairment in pulmonary arteries from diabetic rats

Article Type: Original Article

Section/Category: Cardiovascular diseases

Keywords: proanthocyanidins; oxidative stress; diabetes; pulmonary vascular impairment; antioxidant treatment

Corresponding Author: Professor Angelo Sala, PhD

Corresponding Author's Institution: Università di Milano

First Author: Christian Pinna, PhD

Order of Authors: Christian Pinna, PhD; Paolo Morazzoni; Angelo Sala, PhD

Abstract: Background: Vitis vinifera L. (grape seed extract) is a natural source of proanthocyanidins with antioxidant and free radical-scavenging activities.
Hypothesis: Grape seed extract supplementation may prevent vascular endothelium impairment associated with diabetes mellitus in rat pulmonary artery.
Study design: We evaluated endothelial function of rat pulmonary artery ex-vivo at the intermediate stage (4 weeks) of streptozotocin (STZ)-induced diabetes mellitus. We also evaluated the protective effect of grape seed extract administered daily, beginning the day after diabetes induction, or 15 days after diabetes induction, until the day of sacrifice. In addition, we compared the effect of grape seed extract supplementation with that of vitamin C.
Methods: Rats were made diabetic with streptozotocin (STZ, 65 mg/kg i.v.). Thirty days later rats were sacrificed and pulmonary vessels reactivity and endothelial function compared to that of age-matched healthy animals.
Results: Concentration-response curves to ACh, NE, sodium nitroprusside (NO donor), but not to histamine and iloprost (prostacyclin analog), were significantly altered 4 weeks after STZ-injection. Antioxidant supplementation (3 mg/kg/day) with either vitamin C or grape seed extract, starting the day after diabetes induction, significantly improved vasodilation to ACh and SNP. Norepinephrine-induced contractions were preserved by grape seed extract, but not vitamin C supplementation. Conversely, vitamin C but not grape seed extract showed beneficial effects contrasting the loss of body weight in diabetic animals. Abnormal vascular function was not reversed when antioxidant supplementations were postponed 15 days after the induction of diabetes.
Conclusions: This study provides scientific support for the therapeutic potential of an antioxidant therapy in endothelial impairment associated with diabetes. A daily supplementation of grape seed proanthocyanidins and/or vitamin C given at the earlier stage of disease may have a

complementary role in the pharmacological therapy of diabetes and pulmonary vascular dysfunction.

*Manuscript_highlighted

Proanthocyanidins from *Vitis vinifera* inhibit oxidative stress-induced vascular impairment in pulmonary arteries from diabetic rats

Christian Pinna [a], *Paolo Morazzoni* [b] *and Angelo Sala* [a, c, *]

[a]Department of Pharmacological and Biomolecular Sciences, University of Milan, via Balzaretti, 9, 20133 Milano, Italy; [b] Indena S.p.A. R&D, Milan, Italy;

[c]IBIM, National Research Council, Via Ugo la Malfa 153, 90146 Palermo, Italy

[*]Corresponding author: Angelo Sala, Department of Pharmacological and Biomolecular Sciences, Via Balzaretti 9, 20133 Milano, Italy

ph. +390250318308,

E-mail address: angelo.sala@unimi.it

Abstract

Background: *Vitis vinifera L.* (grape seed extract) is a natural source of proanthocyanidins with antioxidant and free radical-scavenging activities.

Hypothesis: Grape seed extract supplementation may prevent vascular endothelium impairment associated with diabetes mellitus in rat pulmonary artery.

Study design: We evaluated endothelial function of rat pulmonary artery *ex-vivo* at the intermediate stage (4 weeks) of streptozotocin (STZ)-induced diabetes mellitus. We also evaluated the protective effect of grape seed extract administered daily, beginning the day after diabetes induction, or 15 days after diabetes induction, until the day of sacrifice. In addition, we compared the effect of grape seed extract supplementation with that of vitamin C.

Methods: Rats were made diabetic with streptozotocin (STZ, 65 mg/kg i.v.). Thirty days later rats were sacrificed and pulmonary vessels reactivity and endothelial function compared to that of age-matched healthy animals.

Results: Concentration-response curves to ACh, NE, sodium nitroprusside (NO donor), but not to histamine and iloprost (prostacyclin analog), were significantly altered 4 weeks after STZ-injection. Antioxidant supplementation (3 mg/kg/day) with either vitamin C or grape seed extract, starting the day after diabetes induction, significantly improved vasodilation to ACh and SNP. Norepinephrine-induced contractions were preserved by grape seed extract, but not vitamin C supplementation. Conversely, vitamin C but not grape seed extract showed beneficial effects contrasting the loss of body weight in diabetic animals. Abnormal vascular function was not reversed when antioxidant supplementations were postponed 15 days after the induction of diabetes.

Conclusions: This study provides scientific support for the therapeutic potential of an antioxidant therapy in endothelial impairment associated with diabetes. A daily supplementation of grape seed proanthocyanidins and/or vitamin C given at the earlier stage of disease may have a complementary role in the pharmacological therapy of diabetes and pulmonary vascular dysfunction.

Keywords

proanthocyanidins; oxidative stress; diabetes; pulmonary vascular impairment; antioxidant treatment

Abbreviations: GSE, grape seed extract; His, histamine; L-NAME, nitro-L-arginine-methyl ester; NADPH-oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; NE, norepinephrine; NO, nitric oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside; STZ, streptozotocin.

Introduction

The endothelium is a dynamic organ, which regulate vascular tone in response to various hormones and neurotransmitters *via* several vasoactive mediators and primarily nitric oxide (NO) (Sandoo *et al*., 2010). Hyperglycaemia and an excess of reactive oxygen species (ROS) adversely affect endothelial function (Tousoulis *et al*., 2013) and are typical consequences of diabetes mellitus. As a result of endothelial impairment and a reduction of endothelial nitric oxide (eNOS) activity, an increased risk of microangiopathy, peripheral artery diseases (Mcgill *et al*., 2008), cardiovascular and also respiratory dysfunctions (Rosenecker *et al*., 2001) are observed in diabetic patients.

The relationship of diabetes with pulmonary vascular disease had been almost disregarded until recent epidemiological and *in vitro* studies revealed that ROS and diabetes are risk factors for pulmonary arterial hypertension (Moral-Sanz *et al*., 2014; Wong, 2013). Even with the maintenance of near-normal glycaemic values, endothelial dysfunction persists in diabetic patients (Ceriello *et al*., 2007; Dogra *et al*., 2001). Although balancing carbohydrate and insulin is the most important task in managing diabetes, epidemiological studies suggest that regular consumption of foods rich in polyphenols and beverages rich in vitamin C plays a vital role for maintaining weight, and keeping blood low-saturated fats and blood pressure under control, thus reducing the risk of cardiovascular complications (Mccall *et al*., 2009).

Evidence suggests an important role for dietary factors in modulating endothelial function, in particular antioxidant compounds (Bondonno *et al*., 2012), and it has been observed that poor vitamin C blood levels correlate with impaired endothelial function in type-1 diabetic patients (Odermarsky *et al*., 2009).

Flavonoids and proanthocyanidins are the most common antioxidant compounds present in many fruits, vegetables and seeds of Mediterranean diet, and several studies have suggested that grape seed extract confers vascular protection due to the direct effects of its polyphenol content on endothelial cells (Blanch *et al*., 2015; Cui *et al*., 2012; Edirisinghe *et al*., 2008; Feng *et al*., 2010).

A short-term (30 days) administration of streptozotocin, used to induce pancreatic injury, is also used to represent type-1 diabetes, since alterations such as cardiovascular dysfunction are common problems associated with diabetes. Even so, the progression of the disease becomes evident in tissues and organs already after 15 days from diabetes induction (James *et al*., 1994; Toleikis and Godin, 1995; Tong and Cheng, 2002). This study therefore aimed

to evaluate the protective effect of 15 and 30 days of a grape seed extract supplementation, on pulmonary artery responsiveness in diabetic rats treated for 4 weeks with streptozotocin. Results have been compared with those of vitamin C, a physiological non-enzymatic antioxidant present in the blood, which was shown to improve endothelium-dependent responses under certain conditions such as diabetes mellitus or hypertension (Ting, 1996; Taddei et al., 1998).

Materials and methods

Animals and Surgical Procedure:

A total number of 64 male Sprague-Dawley rats weighing 200 - 225 g were used. Animals were assigned at random into four groups of 8 animals each, as follows: (a) age-matched healthy control rats, diabetic rats daily treated with: (b) vehicle, (c) ascorbic acid and (d) grape seed extract, starting on the sixteenth day after the STZ injection and continued daily for the last 15 days. A separate set of animals were also divided into the same groups as mentioned above, but treatment started the same day of diabetes induction.

After a period of not less than 5 days following arrival, rats were made diabetic by a single injection of STZ (60 mg/kg i.v.). Control animals received an equivalent volume of STZ vehicle (0.1 N citrate buffer, pH 4.5). Same day as diabetes induction, 5% glucose was added to the drinking water to prevent hypoglycaemia.

Diabetes induction was considered successful when the blood glucose level was equal or higher than 300 mg/dl (Glucotrend, Roche Diagnostic, Monza, Italy). This was generally the case within 48-72 hours after STZ injection, as described before (Pinna *et al*., 2001). All *in vitro* experiments were carried out the day after last treatment.

Ascorbic acid and grape seed extract were dissolved in distilled water every day at a concentration of 3 mg/kg/day as tested in a study by Pinna *et al*. (2001), and both administered by oral gavage.

Characteristics of plant material:

The standardized extract from *Vitis vinifera* L. grape seed (a brownish-orange powder) was a kind gift of Indena S.p.A. (Milan, Italy). The starting herbal material has been identified against a crude drug standard and authoritative literature source by a botanical analyst. The seeds harvested in September-October from cultivated plants in the European Community were artificially dried and ground to a fine powder. The extraction solvent was water at the temperature of 70-90 °C. Drug extract ratio (DER) was 10-15:1 (native extract). The water content in the dry extract was 5% or less. No excipient or other components (antioxidants/preservatives) for adjustment were added to the powder. The extract was standardized to provide ≥95.0% (accuracy: ±15%) of proanthocyanidins by Folin-Ciocalteu method coupled to UV-visible spectrophotometry, which is a classical method used to determine polyphenol content in plant extracts, as described in the European Pharmacopoeia (Council of Europe, 2007).

The analysis specifically revealed a relatively low amount (8.6%) (by HPLC) of flavane monomers (catechin, epicatechin and epicatechin gallate), and about 91% oligomeric proanthocyanidins (OPC) of which 9% (by HPLC) were of the dimeric type. Pentamers, hexamers, heptamers and their gallates accounted for less than 5% (w/w). HPLC quantitative analysis was carried out as described by Gabetta *et al*. (2000). Briefly, monomers and dimers were quantitated by HPLC-UV at room temp on a SupelcoSil LC-18 column (250×4.6 mm i.d.), particle size 5 µm (Supelco). The solvent system was a linear gradient using MeCN (solvent A) and 0.3% phosphoric acid (solvent B) from 10% A to

20% A in 45 min, then to 60% A in 20 min. The flow-rate was 0.7 ml/min, the UV detector was set at 278 nm and the injection volume was 10 µl.

All experimental procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (Decreto Legislativo N° 26, March 4, 2014, G.U. N° 61 March 14, 2014) and international laws and policies (86/609 EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011).

Ex-vivo experiments on isolated pulmonary artery rings:

Animals were sacrificed with an *i.p.* injection of thiopental sodium (20 mg/kg). Lung and heart were rapidly excised and larger intrapulmonary artery segments were removed and cleaned of adherent parenchimal tissue. Two pulmonary arterial rings (2 mm) from each lung were mounted under 0.8 g of resting tension, in a 5 ml organ bath and perfused with Krebs solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.5 mM glucose, pH 7.4) at 37 °C, continuously bubbled with 95% O_2 and 5% CO_2. Artery rings were connected to isometric force transducers for tension recording (PowerLab, ADInstruments, UK). Following equilibration for 1 h, preparations were precontracted with a submaximal concentration of norepinephrine (NE, 10 µM) to check the vitality of preparations. Norepinephrine was then removed with several washes with Krebs' solution and rings were allowed to rest a further 30 min, until basal tone was restored. Thereafter, cumulative concentration-response curves to the agonists were performed. In between two cumulative concentration-response curves, tissues were washed several times with Krebs' solution and they were allowed to rest for at least 30 min. With the exception of norepinephrine itself, concentration–response curves were done in EC_{60}-norepinephrine-precontracted tissues. The EC_{60} value for norepinephrine used

to evoke precontracted tone, was estimated to be 60 nM for pulmonary artery rings from control rats and 150 nM for diabetic rats, in preliminary experiments.

A different set of diabetic and healthy pulmonary arterial preparations were used to study the effect of grape seed extract itself.

Contractile responses to norepinephrine were measured as increase in tone above baseline (mN/mg tissue), whereas relaxant responses were expressed as percentage of relaxation on EC_{60}-norepinephrine precontracted tissues.

Drugs and Chemicals:

Norepinephrine hydrochloride, Nitro-L-arginine methyl ester hydrochloride, acetylcholine, SNP, indomethacin, ascorbic acid and STZ were purchased from Sigma-Aldrich. Iloprost (SHL 401) was purchased from Shering (Berlin, Germany). All compounds were freshly dissolved in distilled H_2O, except Iloprost, which was purchased as a concentrated water solution.

Statistical Analysis:

All data were expressed as mean±S.E.M. of eight experiments and represent unpaired data. Concentration-response curves were fitted and compared by analysis of variance (ANOVA) using GraphPad Prism3.0. If *P* values were less than 0.05 the treatment affected the response over the tested range of concentration (Ludbrook, 1994). Maximal responses and pD_2 values (-log EC_{50}) for each agonist were compared by one-way ANOVA followed by Tukey's *post hoc* test using GraphPad InStat.

Results

Serum glucose levels and body weight:

A fivefold increase in glycaemia value was observed in untreated STZ-diabetic rats (491± 12 mg/dl), compared to control animals (112±9 mg/dl), (P<0.001). After 30 days of treatment with either grape seed extract or ascorbic acid, glycaemia in treated diabetic rats was still unchanged compared with untreated diabetic rats (Figure 1).

Although there was no difference in body weight prior to diabetes treatment, after 4 weeks of diabetes induction, the body weight of the untreated diabetic group was significantly lower (213±9 g) than that of the healthy group (354±22 g), (P<0.001). Nevertheless, body weight of diabetic rats treated for 30 days with ascorbic acid was significantly higher (266±20 g), (P<0.05) than that of the untreated diabetic rats (Figure 2).

Relaxation to acetylcholine and histamine:

Pulmonary arteries precontracted with (EC_{60})-NE responded to ACh (1 nM – 30 µM) with concentration-dependent vasodilations. The response to ACh, which are endothelium-dependent and mediated by the release of NO, showed a reduced efficacy in diabetic pulmonary arteries. Concentration-response curves to ACh in the control and in the diabetic groups were significantly different (P<0.005), (Figure 3).

Sensitivity to ACh, expressed as the pD_2, also appeared reduced in arterial pulmonary rings from diabetic rats (6.64±0.08) compared to that observed in pulmonary artery from healthy animals (6.86±0.09), but the difference was not statistically significant. Maximal vasodilation was significantly reduced in pulmonary artery from diabetic rats (75.4±2.5%) when compared to that of vessels from healthy animals (98±2.6%), (P<0.001). Pulmonary preparations from diabetic rats supplemented with either grape seed extract or ascorbic acid (3 mg/kg/day) for 30 days, showed a restored response to ACh, and their vasodilatory response to ACh was not different from control tissues. Antioxidant supplementation from day 16 to 30 resulted ineffective.

The relaxation induced by histamine was concentration-dependent (0.1–30 μM), however no significant difference was observed between any of the respective groups (Figure 4).

Relaxation to sodium nitroprusside:

Sodium nitroprusside added cumulatively (0.3 nM – 1 μM) produced on its own complete relaxation of the NE-constricted pulmonary artery rings. Sensitivity to SNP, expressed as the pD_2, was significantly higher ($P<0.01$) in pulmonary artery from untreated diabetic rats (7.94±0.03) than that from healthy animals (7.49±0.04), and the SNP concentration-response curve in diabetic rings was shifted to the left ($P<0.01$), compared to the curve in the controls (Figure 5). Antioxidant supplementation from day 16 to 30 resulted ineffective and did not modify tissue sensitivity to SNP. The pD_2 values in grape seed extract- (7.97±0.04) and in vitamin C-treated group (7.99±0.07) did not differ significantly from that of untreated diabetic group. Antioxidant supplementation from day 1 to 30 restored tissue sensitivity. Significant difference ($P<0.01$) was found between pD_2 values in diabetic rings and those of grape seed extract- (7.57±0.06) or vitamin C-treated animals (7.35±0.05).

Relaxation to iloprost:

The stable prostacyclin analogue iloprost, added cumulatively (0.3 nM – 0.3 μM) to the NE-constricted rings evoked concentration-dependent vasodilations in pulmonary vessel tissues. Tissue sensitivity to iloprost, expressed as the pD_2 were comparable in the diabetic (7.88±0.33) and in the control group (7.80±0.14), as well as maximal responses (E_{max}) being (46.2±3.3%) and (44.1±1.5%) in the diabetic and in the control animals, respectively. Hence, concentration-response curves performed in the untreated diabetic and in the control rings were not significantly different. Ascorbic acid or grape seed extract supplementation for 30 days did not alter iloprost-induced vasodilation in the diabetic group (Figure 6).

Norepinephrine-mediated vascular contraction:

Norepinephrine (1 nM – 10 μM) induced impaired concentration-related contractions in pulmonary artery rings from untreated diabetic rats, compared to controls (Figure 7). Sensitivity to NE, expressed as the pD_2 was significantly reduced ($P<0.05$) in the diabetic (7.15±0.03) compared to control tissues (7.45±0.03). Maximal response was also significantly attenuated ($P<0.01$) in pulmonary artery from diabetic rats (0.98±0.07 mN/mg), compared to that observed in the healthy animals (1.21±0.03 mN/mg) and the two concentration-response curves performed in the untreated diabetic tissues and in the controls were significantly different ($P<0.001$). grape seed extract supplementation administered daily for 30 days protected vascular function from diabetic damage and concentration-response curves performed in the grape seed extract-treated and in the untreated diabetic tissues were significantly different ($P<0.01$). On the contrary, grape seed extract for 15 days or vitamin C supplementation for either 15 or 30 days administered to diabetic animals had no effect on NE-induced responses.

Effect of grape seed extract *on pulmonary arterial rings*:

Grape seed extract added cumulatively (0.1 - 30 μM) produced a slow and sustained relaxation of the NE-constricted pulmonary arterial rings. The relaxation induced by grape seed extract was concentration-dependent and it was not significantly altered by diabetes (Figure 8). Nitro-L-arginine-methyl ester (L-NAME, 10 μM) pretreatment significantly inhibited grape seed extract-mediated vasodilation ($P < 0.01$). Pretreatment with indomethacin (1 μM) plus L-NAME (10 μM) completely blocked vasodilation. On the contrary, ascorbic acid (0.1 - 30 μM) did not induce on its own any response.

Discussion

The present study showed that pulmonary arterial rings from 4-weeks STZ-diabetic rats displayed altered reactivity, resulting in altered vasodilation to ACh, SNP and reduced vasoconstriction to NE. Grape seed extract and vitamin C prevented the observed alterations of vascular responsiveness, when supplemented at the earlier stages of diabetes. An intact vascular endothelium was shown to play an important role in the control of vascular tone (Sandoo *et al*., 2010). Conversely, endothelial dysfunction was associated to the pathogenesis of a large spectrum of human diseases (Su, 2015; Prieto *et al*., 2014) including diabetes (Hamilton and Watts, 2013) and vascular impairment (Tousoulis *et al*., 2013). Substantial evidence also indicated that diabetes mellitus impairs eNOS activity as well as enhances ROS production, thus resulting in diminished NO bioavailability (Su, 2015), increased NO degradation and peroxynitrite formation (Zou, 2007).

Endothelium-dependent vasodilation to ACh was significantly impaired in pulmonary artery from 4-weeks STZ-diabetic rats. Our result was congruent with that of Lopez-Lopez *et al*. (2008) and suggested an altered synthesis or release of NO from vascular endothelium. On the other hand, impaired vascular endothelium and altered vascular reactivity in response to ACh was observed in aortic rings from STZ-diabetic rats after 8-weeks of disease, but not after 2-weeks (Pieper, 1999). Our study showed that either grape seed proanthocyanidins or vitamin C supplementation for 30 days, starting the day of diabetes induction to the day of sacrifice, had a protective effect on pulmonary artery vasodilation to ACh.

On the contrary, both supplementations failed to restore vascular function when postponed 15 days after diabetes induction.

SNP showed increased tissue sensitivity in the untreated-diabetic pulmonary vessels, suggesting the presence of a compensatory mechanism to counteract a reduced endothelial

NO synthesis and release. Lopez-Lopez *et al*. (2008) observed unaltered relaxation to SNP in 6-weeks untreated-diabetic and control pulmonary vessels, however, the discrepancy may be related to fluctuating changes during the course of the disease. Our data also indicated that a daily supplementation of vitamin C or grape seed proanthocyanidins administered immediately after diabetes induction preserved vascular response to SNP.

Prostanoids such as PGI_2, $PGF_{2\alpha}$ and TXA_2 were also involved in the regulation of vascular function. In particular, PGI_2 played a compensatory role in vasodilation when endothelial NO release was impaired (Beverelli *et al*., 1997) and its synthesis increased in rat aorta 8 weeks after diabetes induction (Csanyi et al., 2007). Our result showed that the stable PGI_2 analogue iloprost evoked comparable vasodilations in the control and in the diabetic preparations. However, as in the case of ACh, vascular responses to iloprost might change with the progression of disease.

Contractile response to NE decreased significantly in 4-week untreated diabetic rats. On the contrary, Lopez-Lopez *et al*. (2008) found increased contractile responses to phenylephrine in pulmonary arterial rings from 6-week diabetic rats. The discrepancy may be explained by differences in experimental conditions (longer period of diabetes, different adrenergic agonist used) and in particular by the fact that the response to phenylephrine was expressed as % of KCl response, as in this case an overall impaired contractile capacity would go unaccounted.

NE-induced contractions in the diabetic and in the control preparations were comparable if rats received a daily supplementation of grape seed proanthocyanidins, but not vitamin C, starting at the earlier stages of diabetes.

Several *in vitro* and epidemiological studies suggested an important role of dietary factors with antioxidant properties in preserving endothelial function in a dose dependent manner

(Ghosh and Scheepens, 2009; Mccall *et al*., 2009). While proanthocyanidins received widespread acclaim for their health benefits, several studies indicated limited bioavailability, and no conclusive results have been obtained in clinical studies (Blanch *et al*., 2015). These results may not necessarily indicate that polyphenol-rich foods are not useful. Instead, it should be taken into account that the bio-absorption of polyphenols is very low, and, furthermore, they are rapidly metabolised. Maximum proanthocyanidins plasma level in humans are about 0.15 µM, 1-3 h after polyphenol-rich foods intake (Ghosh and Scheepens, 2009). This concentration is probably too low to have a direct antioxidant protective effect at a vascular level *in vivo,* as suggested by Cohen and Tong (2010), and also to evoke vasodilation *in vitro*. In our experimental conditions, the lower concentration at which grape seed proanthocyanidins evoked vasodilation was 1 µM, in agreement with that observed by Lorenz *et al*. (2004) in rat aortic rings. Furthermore, vasodilation was partially blocked by L-NAME or indomethacin, suggesting that grape seed proanthocyanidins may activate endothelial NO and prostaglandin synthesis.

Our data also showed that grape seed proanthocyanidins extract was as active as vitamin C in restoring ACh and SNP responses, but more active than vitamin C in restoring NE altered contractility, perhaps as a consequence of the synergistic effects among proanthocyanidins contained in the grape seed extract, or among their active metabolites. Both antioxidants resulted ineffective on glycaemia, but vitamin C supplementation contrasted the loss of body weight in diabetic animals.

The timing of antioxidant supplementation also appeared to be as an important parameter while looking at vasoprotection. In our experimental conditions, grape seed proanthocyanidins and vitamin C resulted active in preventing vascular dysfunction only when administered at the very early stages of diabetes.

Not much is known about the mechanisms of action of proanthocyanidins: it was suggested that dietary polyphenols or their metabolites activate, at low concentration, intracellular signalling pathways leading to vascular protection, rather than having a direct antioxidant effect on vascular endothelium. Alternatively, they may interfere or inhibit pro-oxidant enzymatic processes (Ghosh and Scheepens, 2009), and also synergistic effects among polyphenols present in the food or among their active metabolites might increase their efficacy.

Vascular response of pulmonary arteries was altered in the 4-week diabetic rats and grape seeds proanthocyanidins and vitamin C supplementations were useful to preserve endothelial function. Natural antioxidant intake is essential for humans, but dietary vitamin C or the intake of other antioxidant natural compounds may be sub-optimal in oxidant stress exposed, diabetic patients. Although the mechanism by which polyphenols protect endothelial function is still not clear, a daily supplementation of both grape seed proanthocyanidins and vitamin C seems to be a good strategy to prevent or delay diabetes-associated endothelial dysfunction, along with the conventional pharmacological approach.

Acknowledgements

We are grateful to Indena S.p.A. for generously providing a free sample of grape seed extract. The authors thank Dr. Manuela Magnani for the assistance in manuscript preparation.

Conflict of Interest

Angelo Sala and Christian Pinna have no conflicts of interest associated with this publication. Paolo Morazzoni is an employee of Indena S.p.A., a pharmaceutical company

that produces and commercializes the standardized grape seed extract that was used for this research study. This research was completely funded by institutional funds from the Department of Pharmacological and Biomolecular Sciences, University of Milan.

References

Beverelli F, Bea ML, Puybasset L, Giudicelli JF, Berdeaux A. 1997. Chronic inhibition of NO synthase enhances the production of prostacyclin in coronary arteries through upregulation of the cyclooxygenase type 1 isoform. Fundam Clin Pharmacol 1997; 11: 252-9.

Blanch, N., Clifton, P.M., Keogh, J.B. 2015. A systematic review of vascular and endothelial function: Effects of fruit, vegetable and potassium intake. Nutr. Metab, Cardiovasc. Dis. 25, 253-66.

Bondonno, C.P., Yang, X., Croft, K.D., Considine, M.J., Ward, N.C., Rich, L., Puddey, I.B., Swinny, E., Mubarak, A., Hodgson, J.M. 2012. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. Free Radic. Biol. Med. 52, 95–102. doi:10.1016/j.freeradbiomed.2011.09.028

Ceriello, A., Kumar, S., Piconi, L., Esposito, K., Giugliano, D. 2007. Simultaneous control of hyperglycemia and oxidative stress normalizes endothelial function in type 1 diabetes. Diabetes Care 30, 649–654. doi:10.2337/dc06-2048

Cohen, R.A. and Tong X.Y. 2010. Vascular oxidative stress: the common link in hypertensive and diabetic vascular disease. J. Cardiovasc. Pharmacol. 55 (4), 308-16.

Council of Europe. Determination of tannins in herbal drugs. In European Pharmacopoeia, 6th ed.; European Directorate for the Quality of Medicines: Strasbourg, France, 2007; p. A286.

Csanyi G, Lepran I, Flesch T, Telegdy G, Szabo G, Mezei Z. 2007. Lack of endothelium-derived hyperpolarizing factor (EDHF) up-regulation in endothelial dysfunction in aorta in diabetic rats. Pharmacol Rep. 59(4), 447-55.

Cui, X., Liu, X., Feng, H., Zhao, S., Gao, H. 2012. Grape seed proanthocyanidin extracts enhance endothelial nitric oxide synthase expression through 50 -AMP activated protein kinase/surtuin 1-krupple like factor 2 pathway and modulate blood pressure in ouabain induced hypertensive rats. Biol Pharm Bull. 35(12), 2192–7.

1 Dogra, G., Rich, L., Stanton, K., Watts, G.F. 2001. Endothelium-dependent and
2 independent vasodilation studied at normoglycaemia in Type I diabetes mellitus with
3 and without microalbuminuria 44, 593–601.

4 Edirisinghe, I., Burton-Freeman, B., Tissa, K.C. 2008. Mechanism of the endothelium
5 dependent relaxation evoked by a grape seed extract. Clin Sci (Lond). 114(4), 331–7.

6 Feng, Z., Wei, R.B., Hong, Q., Cui, S.Y., Chen, X.M. 2010. Grape seed extract enhances
7 eNOS expression and NO production through regulating calcium-mediated AKT
8 phosphorylation in H2O2-treated endothelium. Cell Biol. Int. 34(10). 1055–61.

9 Gabetta, B., Fuzzati, N., Griffini, A., Lolla, E., Pace, R., Ruffilli, T., Peterlongo, F. 2000.
10 Characterization of proanthocyanidins from grape seeds. Fitoterapia 71, 162–175.

11 Ghosh, D., Scheepens, A. 2009. Vascular action of polyphenols. Mol Nutr Food Res. 53(3),
12 119, 322–31.

13 Hamilton, S.J., Watts, G.F. 2013. Endothelial dysfunction in diabetes: pathogenesis,
14 significance, and treatment. Rev. Diabet. Stud. 10, 133–56.

15 James, G.M., Hodgson, W.C., Davis, E.A., Haynes, J.M. 1994. Attenuated 5-
16 hydroxytryptamine receptor-mediated responses in aortae from streptozotocin-
17 induced diabetic rats. Br J Pharmacol. 111(1), 370-6.

18 Lopez-lopez, J.G., Moral-sanz, J., Frazziano, G., Maria, J., Flores-hernandez, J., Monjaraz,
19 E., Cogolludo, A., Perez-vizcaino, F., Gomez-villalobos, M.J. 2008. Diabetes induces
20 pulmonary artery endothelial dysfunction by NADPH oxidase induction. Am J Physiol
21 Lung Cell Mol Physiol. 295(5), L727-32.

22 Lorenz, M., Wessler, S., Follmann, E., Michaelis, W., Düsterhöft, T., Baumann, G., Stangl,
23 K., Stangl, V., 2004. A constituent of green tea, epigallocatechin-3-gallate, activates
24 endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-
25 dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-
26 dependent vasorelaxation. J Biol Chem. 279(7), 6190-5.

27 Ludbrook, J., 1994. Repeated measurements and multiple comparisons in cardiovascular
28 research. Cardiovasc. Res. 28, 303–11.

29 Maffei Facino, R., Carini, M., Aldini, G., Berti, F., Rossoni, G., Bombardelli,
30 E., Morazzoni, P. 1996. Procyanidines from Vitis vinifera seeds protect rabbit heart
31 from ischemia/reperfusion injury: antioxidant intervention and/or iron and copper
32 sequestering ability. Planta Med. 62(6), 495-502.

33 Maffei Facino, R., Carini, M., Aldini, G., Berti, F., Rossoni, G., Bombardelli,
34 E., Morazzoni, P. 1999. Diet enriched with procyanidins enhances antioxidant activity
35 and reduces myocardial post-ischaemic damage in rats. Life Sci. 64(8), 627-42.

Mccall, D.O., Mcgartland, C.P., Mckinley, M.C., Patterson, C.C., Sharpe, P., Mccance, D.R., Young, I.S., Woodside, J. V, 2009. Dietary intake of fruits and vegetables improves microvascular function in hypertensive subject in a dose dependent manner. Circulation 119, 2153-60.

Mcgill, M., Molyneaux, L., Twigg, S.M., Yue, D.K., 2008. The metabolic syndrome in type 1 diabetes : does it exist and does it matter ? J Diab Compl 22, 18–23.

Moral-Sanz, J., Moreno, L., Cogolludo, A., Perez-Vizcaino, F., 2014. Pulmonary vascular function in insulin resistance and diabetes. Curr. Vasc. Pharmacol. 12, 473–82.

Natella, F., Belelli, F., Gentili, V., Ursini, F., Scaccini, C., 2002. Grape seed proanthocyanidins prevent plasma postprandial oxidative stress in humans. J. Agric. Food Chem. 50, 7720–5.

Nuttall, S.L., Kendall, M.J., Bombardelli, E., Morazzoni, P. 1998. An evaluation of the antioxidant activity of a standardized grape seed extract, Leucoselect. J. Clin. Pharm. Ther. 23(5), 385-9.

Odermarsky, M., Lykkesfeldt, J., Liuba, P., 2009. Poor vitamin C status is associated with increased carotid intima-media thickness, decreased microvascular function , and delayed myocardial repolarization in young patients with type 1. Am J Clin Nutr. 90(2), 447-52.

Pieper, G.M., 1999. Enhanced, unaltered and impaired nitric oxide-mediated endothelium-dependent relaxation in experimental diabetes mellitus: importance of disease duration. Diabetologia 42, 204-13.

Pinna, C., Cignarella, A., Zanardo, R., Bolego, C., Puglisi, L., 2001. Gender differences and antioxidant treatment affect aortic reactivity in short-term diabetic rats Eur.J.Pharmacol. 431, 71–79.

Prieto, D., Contreras, C., Sánchez, A., 2014. Endothelial dysfunction, obesity and insulin resistance. Curr. Vasc. Pharmacol. 12, 412–26.

Rosenecker, J., Höfler, R., Steinkamp, G., Eichler, I., Smaczny, C., Ballmann, M., Posselt, H.G., Bargon, J., von der Hardt, H., 2001. Diabetes mellitus in patients with cystic fibrosis: the impact of diabetes mellitus on pulmonary function and clinical outcome. Eur. J. Med. Res. 6, 345–50.

Sandoo, A., Veldhuijzen van Zanten, J.C.S., Metsios, G.S., Carroll, D., Kitas, G.D. 2010. The Endothelium and Its Role in Regulating Vascular Tone. Open Cardiovasc. Med.J. 4, 302-12.

Su, J.B. 2015. Vascular endothelial dysfunction and pharmacological treatment. World J Cardiol. 7 (11), 719-41.

Taddei, S., Virdis, A., Ghiadoni, L., Magagna, A., Salvetti, A. 1998. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. Circulation. 97, 2222-9.

Ting, H.H., Timimi, F.K., Boles, K.S., Creager, S.J., Ganz, P., Creager, M.A. 1996. Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. J. Clin. Invest. 97, 22-8.

Toleikis, P.M., Godin, D.V. 1995. Alteration of antioxidant status in diabetic rats by chronic exposure to psychological stressors. Pharmacol Biochem Behav. 52(2), 355-66.

Tong, Y.C., Cheng, J.T. 2002. Alteration of M(3) subtype muscarinic receptors in the diabetic rat urinary bladder. Pharmacol. 64(3), 148-51.

Tousoulis, D., Papageorgiou, N., Androulakis, E., Siasos, G., Latsios, G., Tentolouris, K., Stefanadis, C., 2013. Diabetes Mellitus-associated vascular impairment - Novel circulating biomarker and therapeutic approaches. J.Am.Coll.Cardiol. 62 (8), 667-76.

Vigna, G.B., Costantini, F., Aldini, G., Carini, M., Catapano, A., Schena, F., Tangerini, A., Zanca, R., Bombardelli, E., Morazzoni, P., Mezzetti, A., Fellin, R., Maffei Facino, R., 2003. Effect of a standardized grape seed extract on low-density lipoprotein susceptibility to oxidation in heavy smokers. Metabolism. 52, 1250–7.

Wong, C., 2013. Reactive Oxygen Species and Antioxidants in pulmonary hypertension. Antioxid Redox Signal. 18(14), 1789-96.

Zou, M.H., 2007. Peroxynitrite and protein tyrosine nitration of prostacyclin synthase. Prostaglandins Other Lipid Mediat. 82(1-4):119-27.

Figure Legends

Figure 1. Glycaemia (mg/dl) in control, untreated diabetic, grape seed extract- (GSE) and vitamin C-treated for 30 days. $^{*}P<0.001$: control vs. diabetic.

Figure 2. Body weight (g) in control, untreated diabetic, grape seed extract- (GSE) and vitamin C-treated for 30 days. $^{*}P<0.001$: control vs. diabetic; $^{+}P<0.05$: vitamin C vs. diabetic.

Figure 3. Cumulative concentration-response curves for acetylcholine in pulmonary arterial rings from control, untreated diabetic, grape seed extract- (GSE) and vitamin C-treated for 15 days (starting on day 16 after diabetes induction) and for 30 days. Data represent the mean % of vasodilation of NE-precontracted rings±S.E.M. (n=8 in each group). Statistical significance for curves: $^{*}P<0.001$: control vs diabetic; $^{+}P<0.01$: vitamin C (30 days) vs. diabetic; and GSE (30 days) vs. diabetic.

Figure 4. Cumulative concentration-response curves for histamine in pulmonary arterial rings from control, diabetic untreated, grape seed extract- (GSE) and vitamin C-treated for 30 days. Data represent the mean % of vasodilation of NE-precontracted rings±S.E.M. (n=8 in each group).

Figure 5. Cumulative concentration–response curves for SNP in pulmonary arterial rings from control, diabetic untreated, grape seed extract- (GSE) and vitamin C-treated for 15 days (starting on day 16 after diabetes induction) and for 30 days. Data represent the mean % of vasodilation of NE-precontracted rings±S.E.M. (n=8 in each group). Statistical significance for curves: $^{*}P<0.01$: control vs diabetic; vitamin C (30 days) vs. diabetic; GSE (30 days) vs. diabetic.

Figure 6. Cumulative concentration–response curves for iloprost in pulmonary arterial rings from control, diabetic untreated, grape seed extract- (GSE) and vitamin C-treated for 30 days. Data represent the mean % of vasodilation of NE-precontracted rings±S.E.M. (n=8 in each group).

Figure 7. Cumulative concentration–response curves for norepinephrine in pulmonary arterial rings from control, diabetic untreated, grape seed extract- (GSE) and vitamin C-treated for 15 days (starting on day 16 after diabetes induction) and for 30 days. Contractile tension is expressed as mN/mg weight tissue, and data are shown as the means±S.E.M. (n=8 in each group). Statistical significance for curves: $^{*}P<0.001$: control vs untreated diabetic; $^{+}P<0.01$: GSE (30 days) vs. diabetic.

Figure 8. Cumulative concentration–response curves to grape seed extract (GSE) in pulmonary arterial rings from control, untreated diabetic, control+L-NAME, control+L-NAME+indomethacin. Data represent the mean % of vasodilation of NE-precontracted rings±S.E.M. (n=8 in each group). Statistical significance for curves: $^{*}P<0.01$: control vs control+L-NAME. Lower panel: an original tracing showing GSE-induced relaxation in control tissue.

Figure 1

Figure 1

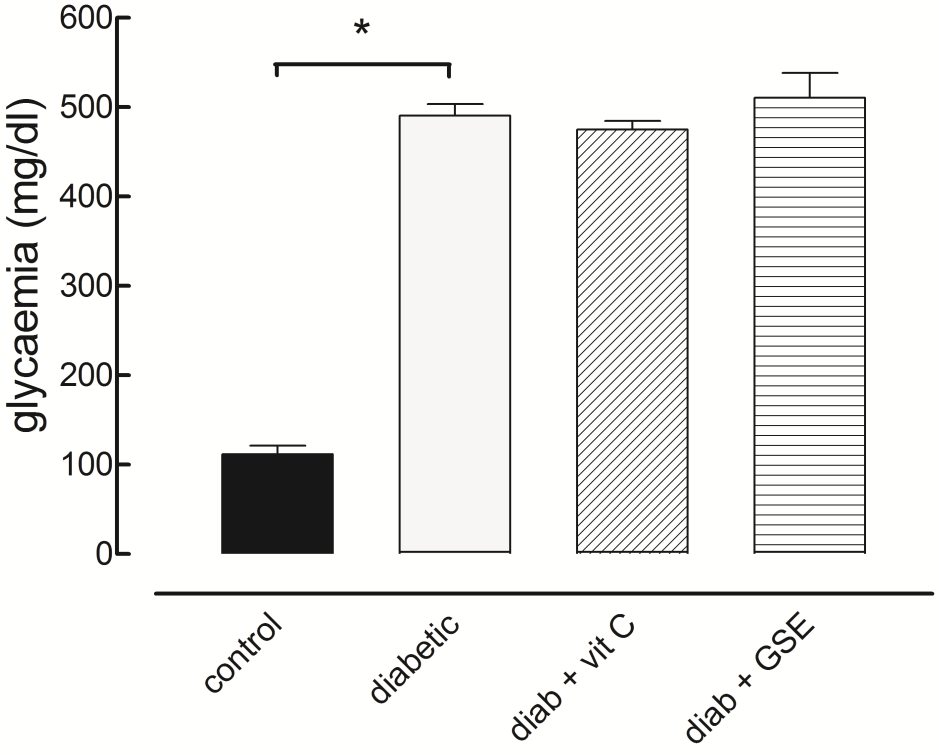


Figure 2

Figure 2

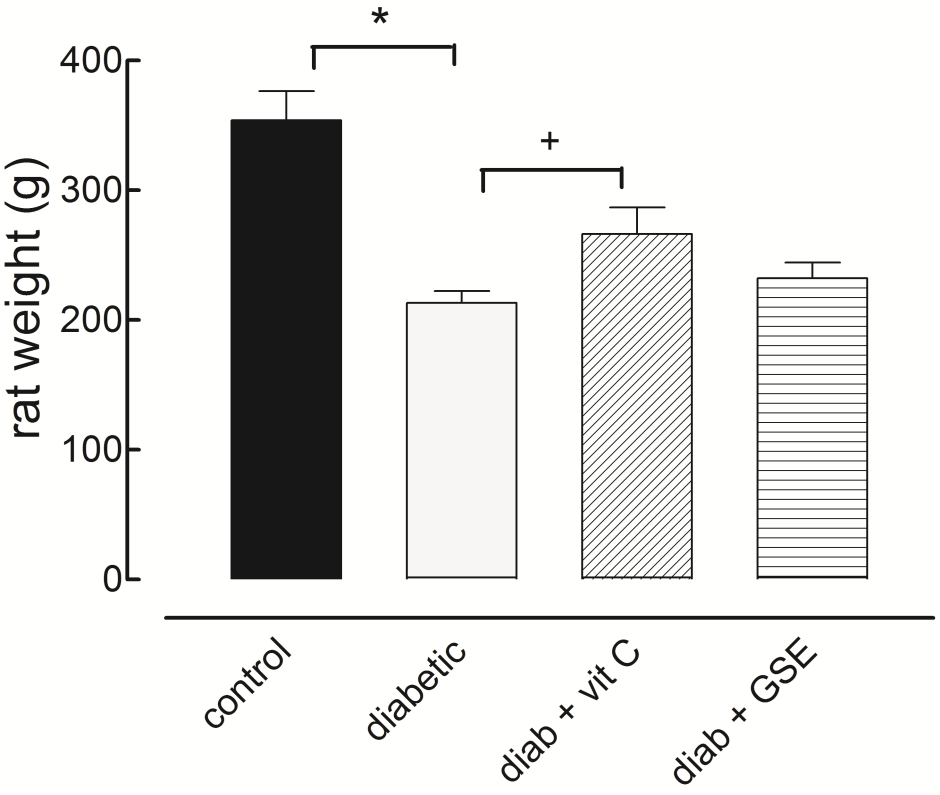


Figure 3

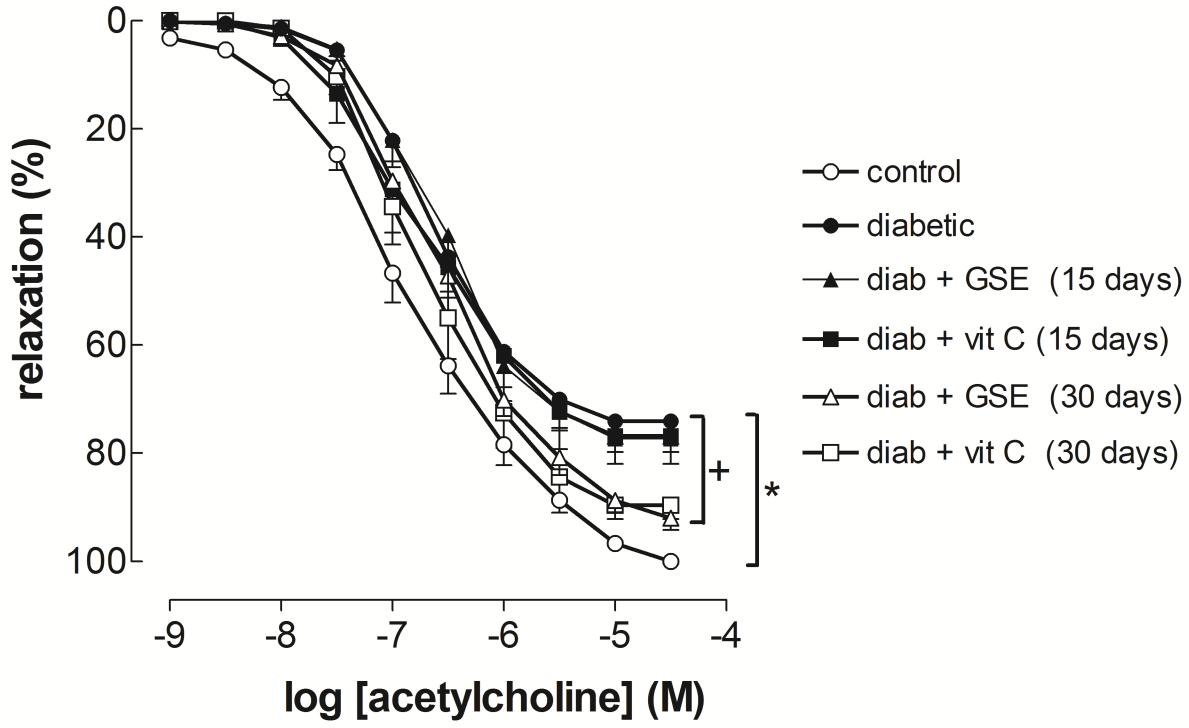


Figure 3
0
20
40
60
80
100
relaxation (%)
-9
-8
-7
-6
-5
-4
log [acetylcholine] (M)
+
*
control
diabetic
diab + GSE (15 days)
diab + vit C (15 days)
diab + GSE (30 days)
diab + vit C (30 days)

Figure 4

Figure 4

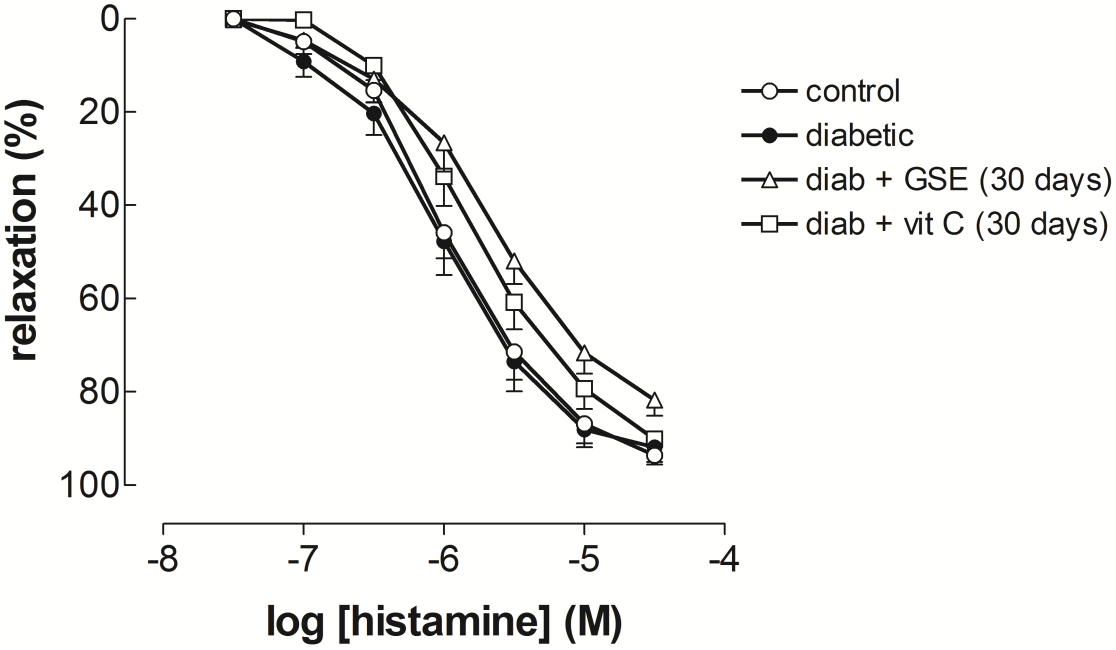


Figure 5

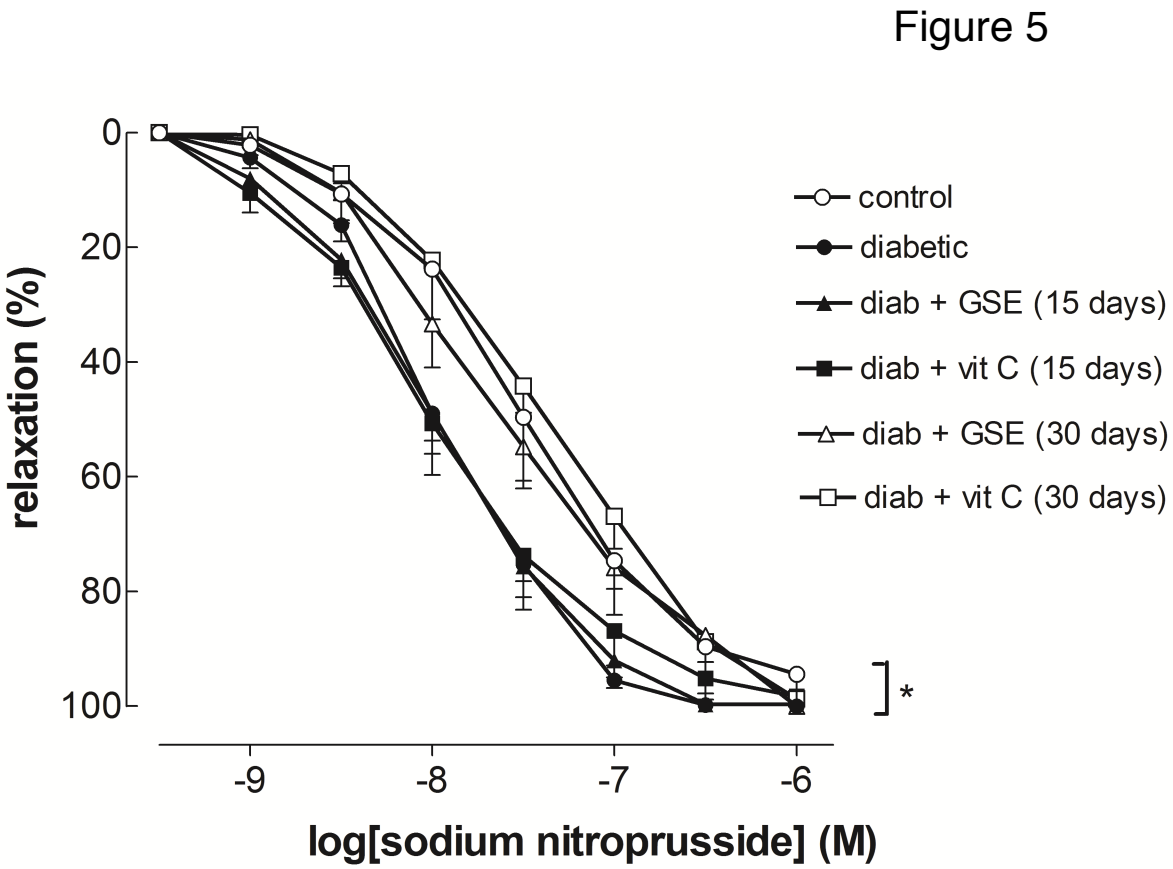


Figure 5
0
20
40
60
80
100
relaxation (%)
-9
-8
-7
-6
log[sodium nitroprusside] (M)
control
diabetic
diab + GSE (15 days)
diab + vit C (15 days)
diab + GSE (30 days)
diab + vit C (30 days)
*

Figure 6

Figure 6

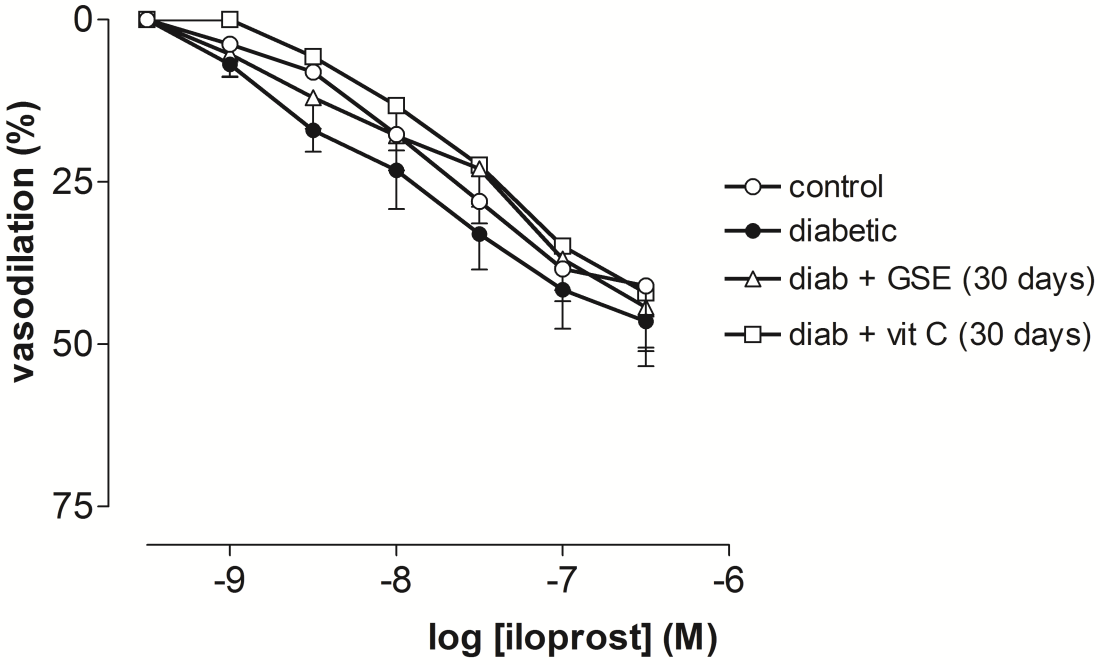


Figure 7

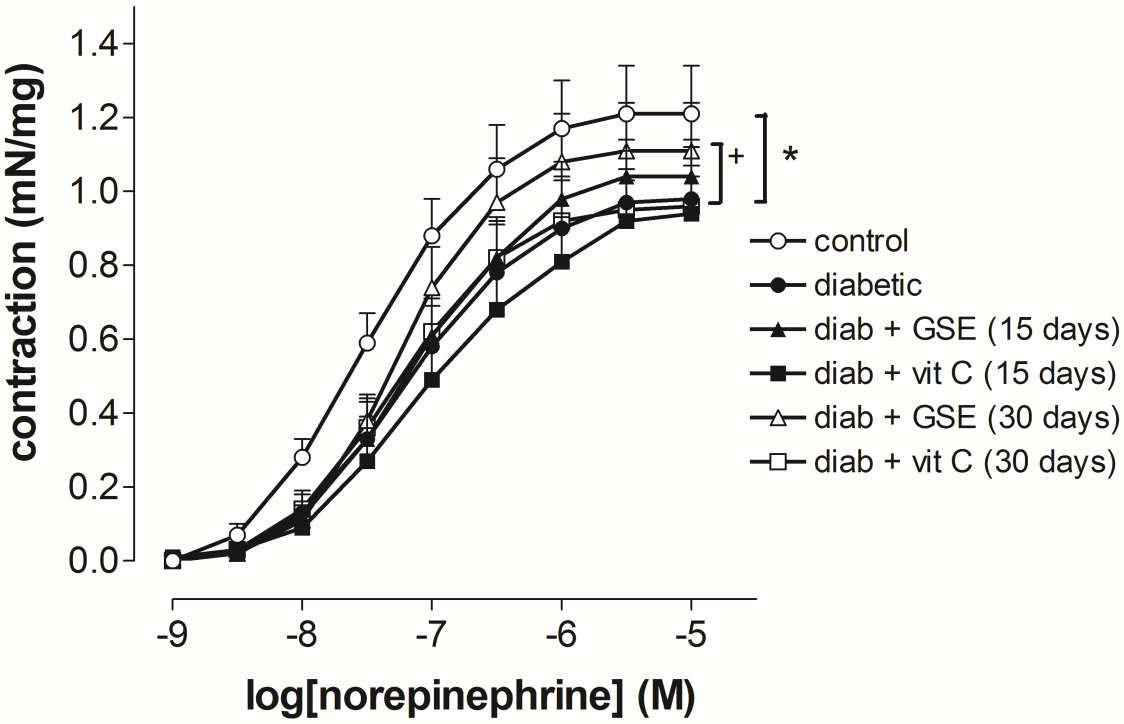
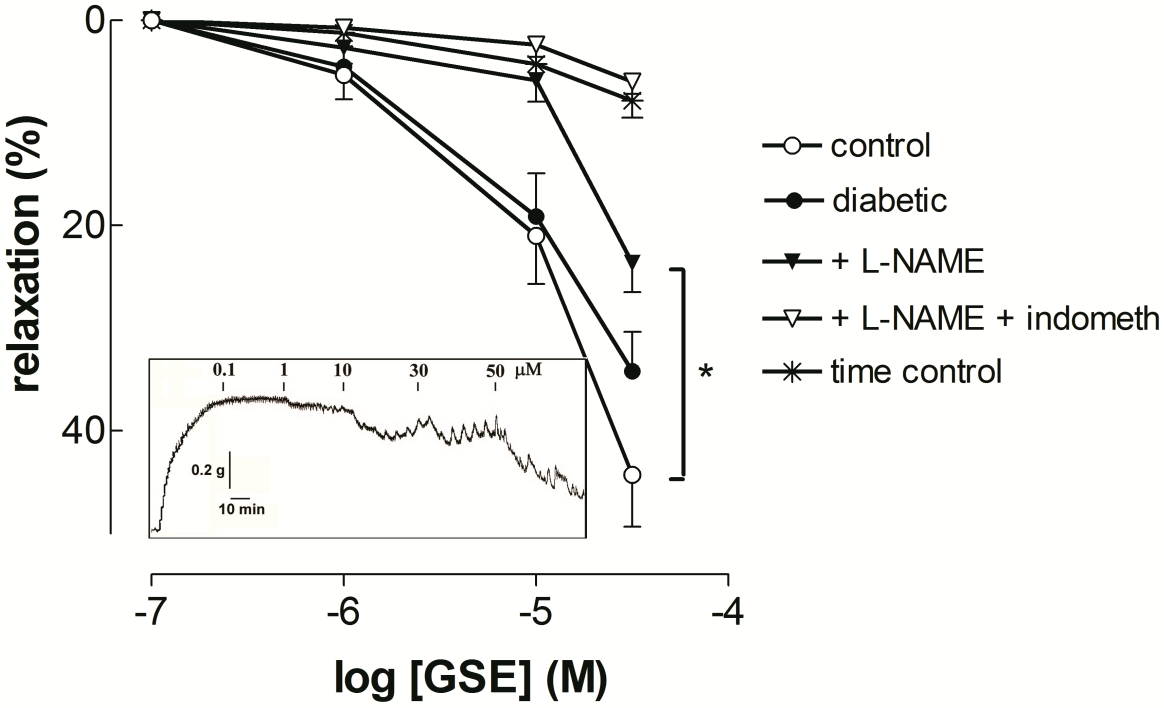
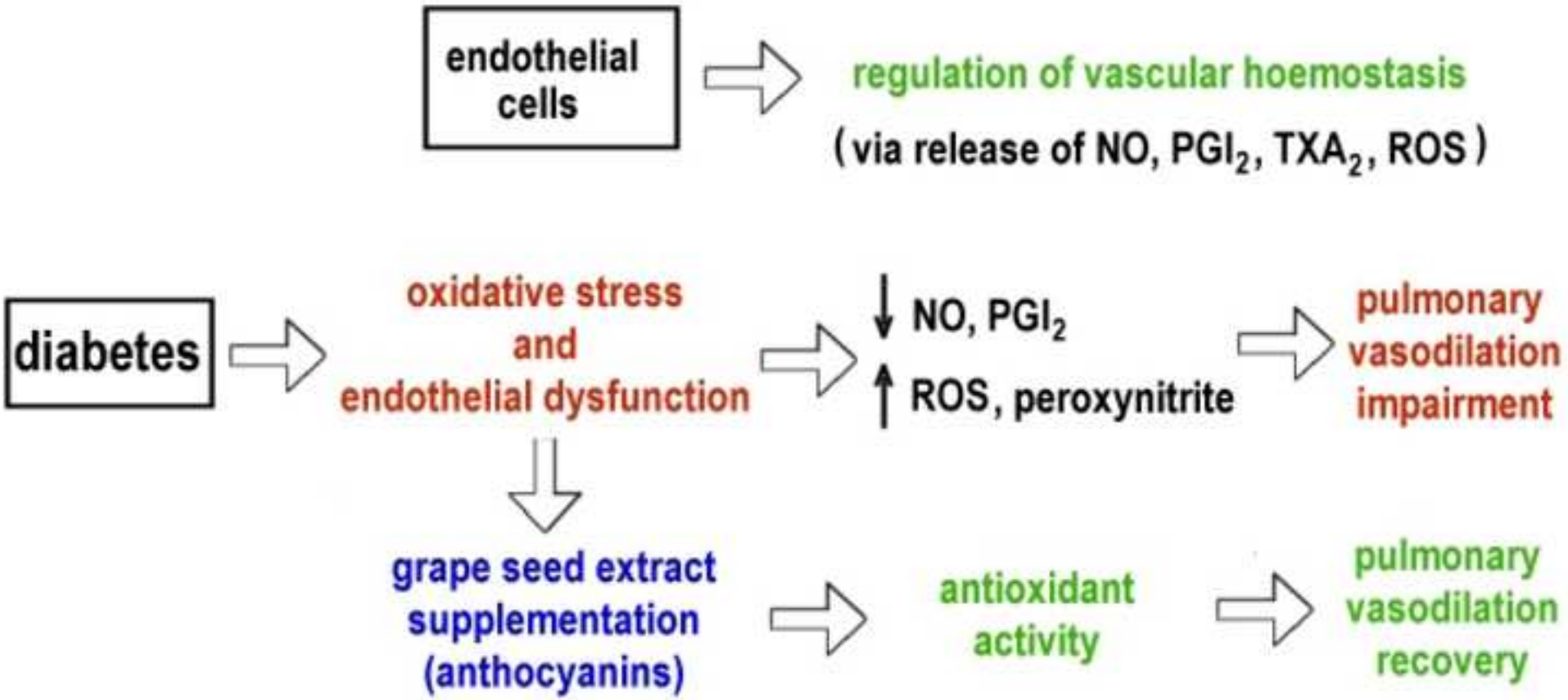

contraction (mN/mg)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-9
-8
-7
-6
-5
log[norepinephrine] (M)
+
*
control
diabetic
diab + GSE (15 days)
diab + vit C (15 days)
diab + GSE (30 days)
diab + vit C (30 days)

Figure 8


0
20
40
relaxation (%)
0.1
1
10
30
50 µM
0.2 g
10 min
*
-7
-6
-5
-4
log [GSE] (M)
control
diabetic
+ L-NAME
+ L-NAME + indometh
time control

Graphical Abstract